Chestnut and lemon balm based ingredients as natural preserving agents of the nutritional profile in matured "Serra da Estrela" cheese

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Abstract

Chestnut flowers and lemon balm plants and their decoctions were incorporated into "Serra da Estrela" cheese, to assess their potential to preserve its nutritional properties and provide new foodstuffs. The analyses were carried out after the normal ripening period of 1 month and after 6 months of storage. The most abundant nutrients were proteins and fat. The most abundant minerals were Ca and Na, while C16:0 and C18:1 were the main fatty acids. Saturated fatty acids were the most abundant, followed by the monounsaturated. Moisture seemed to be lower in the samples incorporated with the plants. The dried plants when incorporated seemed to be more efficient as preservers then the decoctions, although these better preserved the proteins. These plants can be regarded as promising natural preservers in foodstuffs cheese, given the preservation of key parameters and the slight impact on the nutritional value.

Keywords: *Castanea sativa* Mill.; *Melissa officinalis* L.; nutritional composition; fatty acids; cheese

1. Introduction

The ever-growing food industry is avid for innovation in foodstuffs in order to create new markets and stimulate purchasing. For a few years, consumers have been gaining interest in healthy, functional and bioactive foodstuffs, which are prepared with the least additives possible, and preferably with natural ingredients (Carocho, Barreiro, Morales, & Ferreira, 2014a). This happens due to the concern with consumers' health and with healthier way of living. To adapt to these behavioural changes in consumption, the food industry is searching for innovative, healthy and cheap food products. Plants are an endless resource of bioactive compounds and their use in the diet is increasing, especially in developed countries. Among the plants, flowers are starting to be used as food for their appearance, taste and beneficial properties (Mlcek & Rop, 2011). Chestnut flowers (*Castanea sativa* Mill.) have an outstanding antioxidant capacity, also displaying antimicrobial and antitumor effects. This activity could be related to their phenolic fraction, which could also contribute to other described medicinal effects, namely as mucolytic, antispasmodic and anti-dysenteric treatment (Neves, Matos, Moutinho, Queiroz & Gomes, 2009; Carocho, et al., 2014b; Carocho, Barros, Bento, Santos-Buelga, Morales, & Ferreira, 2014c). Other plants, like lemon balm (*Melissa officinalis* L.), also display many medicinal effects, especially when consumed as infusion or decoction. These effects encompass expectorant, digestion relief, ease of headaches and rheumatism, prevention of neurodegenerative diseases, antitumor, antiproliferative, anticholinesterase, antioxidant and anti-Alzheimer properties among many others (Carnat, Carnat, Fraisse, & Lamaison, 1998; Salah, & Jäger, 2005; Martins et al., 2012; Barros, Dueñas, Dias, Sousa, Santos-Buelga, & Ferreira, 2013; Pereira et al., 2014; Carocho et al., 2015a). Moreover, both these plants could act as antioxidants and antimicrobials in foodstuffs, acting as natural additives within the foodstuff, as well as aiding health benefits to potential consumers. The food industry is

spending increasing efforts to find natural compounds and/or plant extracts to be incorporated into food in order to substitute chemical additives in its formulations, mainly due to controversies related to some of these compounds that could involve them in undesirable effects towards human health (Carocho, Morales, & Ferreira, 2015b). Although the incorporation of plant extracts in foodstuffs has been carried out previously (McCarthy, Kerry, Kerry, Lynch, & Buckey, 2001; Stojković et al., 2013; Reihani, Tan, Huda, & Easa, 2014), including by our research group (Carocho, Barreira, Bento, Morales, & Ferreira, 2014d; Carocho et al., 2015c; Carocho, Barreira, Antonio, Bento, Morales, & Ferreira, 2015d), it is the first time that is done in "Serra da Estrela" cheese in order to determine the variation of the nutritional profile along storage time. Previously, these same plants were used to functionalize "Serra da Estrela" cheese, bringing them antioxidant activity (Carocho et al., 2015d). This Portuguese delicacy is the most famous cheese produced in the country, and is appreciated worldwide. It is only manufactured with raw ewe's milk, salt and milk thistle flower for coagulation, and it is preferentially consumed as a soft cheese, with an average maturation of 1 month, although some consumers prefer to consume it as hard cheese after at least 6 months of storage. Despite some published studies on "Serra da Estrela" cheese that focus mainly on the microbial flora in the cheese as well as the changes in some nutrients along ripening time (Partidário, Barbosa, & Boas, 1998; Dahl, Tavaria, & Malcata, 2000; Tavaria, Franco, Carballo, & Malcata, 2003; Tavaria, Reis, & Malcata, 2006; Macedo, Tavares, Malcata, 2004), the present work evaluates the effects of natural additives (chestnut flowers and lemon balm dry material and decoctions) on the nutritional properties of cheese along its maturation.

2. Materials and Methods

2.1. Plant material and natural additives

Chestnut flowers, *Castanea sativa* Mill., belonging to the cultivars Judia and Longal were obtained in June 2013 from Oleiros in Bragança, Portugal (41º51' 02''N, 6º49'54''W). After lyophilisation (FreeZone 4.5, Labconco, Kansas, USA) they were milled down to a fine powder. The decoction procedure was previously described by Carocho et al. (2015d). Briefly, 5 grams of the chestnut flower powder was added to 1 L of cold water and heated until it reached its boiling point; it was maintained at this temperature for 5 minutes. Then, the heat was turned off and left for another 5 minutes. After filtration through a Whatman Nº4 filter the decoction was further frozen and lyophilized. The same procedure was carried out for dried lemon balm, *Melissa officinalis* L., stems and leaves, which were provided by the company "Mais Ervas" (Alfândega da Fé, Portugal). Both dry material and lyophilized decoctions of chestnut flowers and lemon balm were used as natural additives that were incorporated in "Serra da Estrela" cheese.

2.2. Cheese production

The cheese samples used in the assays were produced in the certified cheese factory "Queijos Casa Matias", based in Seia, Portugal. The ewe milk (breed Churra Mondegueira) was collected in the morning and brought to the facility under a constant temperature of 5ºC and a pH of 6.88. The milk was placed in an automated mixer reservoir, which was attached to an automated cheese producing machine. Salt (30 g/L of milk) and milk thistle extract (*Cynara cardunculus* L.) (0.4 g/L of milk) were added to the milk and left to coagulate. After the milk coagulation (1 h), the cheese was collected from the reservoir and placed in molds, which were slightly pressed to remove the serum. Then, the molds are placed in a conveyor belt to be collected by personnel for a second pressing at higher pressure. After 2 hours of a second pressing the cheese is embalmed with a cloth around its sides to maintain its format and placed in maturing chambers. The first chamber had a

relative humidity of 95 to 100% and a constant temperature ranging from 7 to 9ºC. The cheeses were kept here for fifteen days, after which they were transferred to the second chamber, kept between 80 to 82% of relative humidity and 11 to 13 ºC for another fifteen days. The cheeses were washed with water every second week to remove exterior contamination.

2.3. Incorporation of the natural additives

The dried plants and their respective decoctions were added to the cheese between the two pressings, by manually mashing them, adding the natural additives and replacing them into the molds for the second pressing. After incorporation, the cheese followed all the normal steps of production. Five lots of cheese were produced: 1- cheese samples incorporated with dried chestnut flowers, 2- cheese samples incorporated with dried lemon balm, 3- cheese samples incorporated with decoction of chestnut flower, 4- cheese samples incorporated with decoction of lemon balm, and 5- cheese with no incorporation, control lot. In order to determine the quantity of dried plant or decoction to be added to the cheese, the EC_{50} value of the DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant assay was used. The EC_{50} of chestnut flower was 99.47 µg/mL, as reported previously (Carocho et al., 2014d). This value was adjusted to the quantity of milk used for each cheese, resulting in the addition on 248 mg of chestnut decoction per cheese. For the lot incorporated with dried flowers, the decoction extraction yield (31%) was used. Therefore, 31% corresponded to the EC_{50} of the decoction (99.47 µg/mL), and 319 µg/mL corresponded to 100%. Once again, by adjusting the amount of milk used per cheese, 799 mg of dried flower was added to each one. For the other natural additive (lemon balm), the same calculations were used (EC_{50} – 60 µg/mL; yield 38.9%), thus, 380 mg of plant decoction

per cheese for one lot, and 368 mg of dried plant per cheese for the other lot (Carocho et al., 2015a).

After the maturation period (1 month), all the cheese samples were collected from the company and brought to the laboratory under controlled temperature. Two cheeses of each lot were immediately processed, being peeled, cut into small cubes, frozen, lyophilized and milled down, while the other two cheeses from the lot were kept in refrigeration (5ºC) during a storage period of 6 months and then processed in the same manner.

2.4. Standards and reagents used in the laboratorial analyses

Micro (Fe, Cu, Mn and Zn) and macroelements (Ca, Mg, Na and K) standards (> 99% purity), as well as $LaCl_2$ and CsCl (> 99% purity) were purchased from Merck (Darmstadt, Germany). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers. Sulfuric, hydrochloric and nitric acid were obtained from Fisher Scientific (Waltham, MA, USA) and nitric acid was purchased from Sigma (ST. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.5. Evaluation of nutritional profile

2.5.1. Proximate composition

The proximate composition was calculated based on moisture, proteins, fat and ash, relying on the official AOAC procedures (AOAC, 2012). Moisture was determined by desiccation at constant weight at 100 ± 2 °C. Total protein content (N × 6.38) was calculated as nitrogen content by the Kjeldahl method, while crude fat relied on the extraction of dried samples with petroleum ether using a Soxhlet apparatus. The ash content was determined

by incineration at 550 ± 15 °C. Finally, carbohydrates were calculated by difference, but due to not being detected, they were considered zero.

2.5.2. Fatty acids

Fatty acids were determined by gas chromatography. The equipment consisted of a gas chromatograph (GC) (DANI 1000, Contone, Switzerland) coupled to a split/splitless injector and a flame ionization detector (FID) as previously described (Barros, Oliveira, Carvalho, & Ferreira, 2010). The identification was carried out by comparing the relative retention times of the fatty acids methyl esters of the samples (FAME) to commercial standards. The quantification was achieved through CSW 1.7 (DataApex 1.7, Prague, Czech Republic). The results were expresses in relative percentage of each fatty acid.

2.5.3. Mineral composition: Macro and microelements

The procedure for the total mineral content followed the 930.05 AOAC methodology. The samples were subject to incineration at 550 ± 15 ºC, with the resulting residue being dissolved in HCl and HNO_3, and finally adjusting the volume with distilled water. Microelements (Fe, Zn and Cu) were measured directly, while the macroelements (Ca, Mg, Na and K) were diluted at a 1/10 reason to avoid interferences. The methodology followed was previously reported by Fernández-Ruiz, Olives, Cámara, Sánchez-Mata, & Torija, (2011), in which Analyst 200 Perkin Elmer (Perkin Elmer, Waltham, MA, USA) atomic absorption spectroscope (AAS) with air/acetylene flame was used. The absorbances were compared with responses >99.9% pure analytical standard solutions for AAS made with $Fe(NO_3)_3$, $Zn(NO_3)_2$, NaCl, KCl, $CaCO_3$ and Mg band. The results were expressed in mg/100g of fresh weight.

2.6. Statistical analysis

The five lots of cheese (control, decoction of chestnut flower, decoction of lemon balm, dried chestnut flowers and dried lemon balm) were labelled, and two samples of each were subjected to immediate analysis (after 1 month maturation), while others were stored at a constant temperature (3.7±1.6 ºC) for 6 months, after which they were analysed too. All the analysis were carried out in triplicates, and the data were expressed as mean±standard deviation, maintaining the decimal places allowed by the magnitude of the standard deviation. An analysis of variance (ANOVA) with type III sums of squares was performed using the GLM (General Linear Model) procedure of the SPSS software. The dependent variables were analyzed using 2-way ANOVA, with the factors "natural additive" (NA) and "storage time" (ST) for each plant species independently. When a statistically significant interaction (NA×ST) was detected, the two factors were evaluated simultaneously by the estimated marginal means plots for all levels of each single factor. Alternatively, if no statistical significant interaction was verified, means were compared using Tukey's honestly significant difference (HSD) multiple comparison test to evaluate the NA effect, or by a *t*-student test to assess the effect of ST.

Besides comparing the effects of different natural additives and storage time for each plant species, the results for all parameters were compared for both plants simultaneously aiming to (i) verify which plant species was the most suitable, independently of using dried plant or decoction, and also to (ii) find which type of natural additive constitute the best solution, independently of the plant species. A stepwise technique, *linear discriminant analysis (LDA)*, using the Wilks' λ method with the usual probabilities of *F* (3.84 to enter and 2.71 to remove), was applied for variable selection. This procedure uses a combination of forward selection and backward elimination procedures, where before selecting a new variable, it is verified whether all variables previously selected remain significant

(Palacios-Morillo, Alcázar, Pablos, & Jurado, 2013). With this approach, it is also possible to identify the significant variables that contribute most to the possible discrimination of a natural additive (dry material or decoction) or plant species (chestnut or lemon balm). To verify which canonical discriminant functions were significant, the Wilks' λ test was applied. A leaving-one-out cross-validation procedure was carried out to assess the model performance.

All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., Armonk, NY, USA).

3. Results and Discussion

3.1. Effects on nutritional parameters

As explained by Carocho et al. (2014b, c) chestnut flowers have excellent antioxidant and antimicrobial capacity, and could be used to functionalize food matrices. (Carocho et al., 2014d, Carocho et al., 2015d). Despite no studies on the incorporation of lemon balm in foods have been previously reported, the antioxidant activity of this plant has also been described by our research group (Carocho et al., 2015a). Therefore, dried material and lyophilized decoctions of both plant species were used as natural additives in "Serra da Estrela" cheese, in order to evaluate their preserver effects along storage time.

Tables 1-3 report the proximate composition, mineral composition and fatty acid profile for all the assayed samples, after 1 month of ripening, and after 6 months of maturation. The upper section of each table refers to the dried chestnut flowers and the bottom one to lemon balm. Each of these sections is divided into two parts; natural additive type (NA) (dried plant or decoction) and storage time (ST) (with 1 month and 6 months), both including the control cheese samples (none natural additive). In both cases, the results are presented as the mean value of each NA for both storage times, and also the mean value of

ST with all the different natural additives. This approach was used to help identifying the optimal ST independently of the natural additive used in the cheese, and also the best natural additive independently of the storage to which the cheese was subject. Thus, the standard deviations should not be regarded as a measure of accuracy of the applied methodologies, since they encompass the results obtained from the samples prepared in different conditions (variation of the non-fixed factor: ST or NA). The interaction between both effects was also evaluated. Every time that a significant interaction was found (p<0.050), no multiple comparisons could be performed. In those cases, the influence of each individual factor was drawn from the estimated marginal means (EMM) plots.

As expected, the samples lost moisture during the 6 months of storage time (as seen in **Table 1**), which could explain the increase in some components during time. **Table 1** reports the proximate composition, and regarding the cheese samples with chestnut flowers, a significant interaction was found for all parameters (NA × ST < 0.05), therefore some tendencies were extracted from the EMM plots. Still, regarding the NA, the effect of each individual factor was not significant for moisture, fat, protein and energy, contrarily to the observed for the ash content. Furthermore, concerning the ST, the effect of each individual factor was significant for all the parameters. Regarding lemon balm, the behaviour was similar to chestnut flowers, with a significant interaction for all the parameters, with tendencies being conveyed through the EMM. These plots showed that moisture decreased in all cheese types after the 6 months of storage, which made all the other constituents of the cheese to increase. Still, the cheese samples with the natural additives lost a higher quantity of moisture overtime. This is a very interesting feature carried out by the plants, because the decrease of moisture helps preserve the organoleptic and structural shape of the cheese, due to the growth inhibition of proteolytic and other bacteria that cause defect and alterations in cheese, and in some instances, be harmful for

consumption (Segat et al., 2014). Furthermore, by decreasing the moisture the contamination risk is lowered, and the cheese can achieve the state of being considered, "old" cheese, faster (very appreciated by consumers, and with a higher value), and therefore reduce the time of ripening needed to achieve this state. A higher amount of proteins was detected in the cheese added with the natural additives (regardless of being with decoction or dried plant), which is an interesting fact. The plants could have inhibited the development of proteolytic bacteria, which reduced the destruction of the protein fraction in the 1 month of ripening. The total fat seemed to be higher after the 6 months of maturation, although appeared to be lower in the cheese incorporated with the dried plant (**Figure 1A to 1C**). This is due to the reduction in moisture over the course of the 6 months, and the fact that the results were expressed in g/100 of fresh weight.

Table 2 reports the mineral composition of the cheese. Calcium, magnesium, sodium, potassium, iron and copper were the macro and micro minerals detected in the cheese samples. Calcium and sodium were the most abundant minerals, while copper was present in very fewer quantities. Once again, for both plants, a significant interaction was detected for all minerals, allowing tendencies to be drawn from the EMM. Regarding cheese samples with chestnut flowers and NA, the effect of each factor was significant for Ca, Mg, Fe and Zn, although Fe was the only mineral where both factors were not significant, with regard to ST. The same behaviour was reported for lemon balm. Some minerals seemed to duplicate over time, but this could be explained by the loss of moisture of all the cheeses during storage, aiding to the sensibility of the method and the statistical treatment. The EMM plots show that the cheese incorporated with the decoctions showed more sodium after 6 months, but the cheese with the dried plant had less calcium in the beginning of the storage period (**Figure 1D** and **1E**).

Table 3 reports the detected individual fatty acids and the total percentages of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). The most abundant fatty acids in the cheese samples were C18:1 and C16:0, and the SFA prevailed over the MUFA, being PUFA the least abundant. Regarding cheese samples with chestnut flowers, significant interactions were detected for C4:0, C6:0, C8:0, C14:0, C16:0 C16:1, C17:0 and C18:2, therefore, some tendencies were retrieved from the EMM plots. Regarding NA, they had significant interactions for C12:0, C14:0, C15:0, C16:0, C16:1, C17:0, C18:1, C18:2, SFA, MUFA and PUFA, while ST was only significant for C4:0 and C6:0. For the cheese samples added with lemon balm, significant interactions were detected for C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:1, C18:2, C18:3, SFA, MUFA and PUFA, while ST was only significant for C6:0. It is noticeable that the use of decoctions and dried plants as natural additives had higher effects on the fatty acids profile than the storage time. The EMM plots reveal that C16:0 and SFA showed very little change among the different cheese types, while C18:1 was more abundant in the cheese samples with the dried plants. Being this fatty acid a monounsaturated molecule, and one of the most abundant ones in this cheese, its conservation along the 6 months is desirable. The cheese samples with the decoctions seemed to show less MUFA, while the ones with the dried plants had more, once again these molecules were preserved by the dried plants, which proves their efficacy as preservatives, promoting the beneficial effects of these unsaturated molecules. In terms of PUFA, the cheese incorporated with dried plants showed more at 1 month. There was very little change in these fatty acids during the storage period. Given that this is the least abundant fraction of fatty acids, with only two significant molecules, it was expected that very slight changes would be detected (**Figure 1F-1J**).

3.2. Linear discriminant analysis (LDA)

In the former section, the alterations resulting from the incorporation of chestnut flowers or lemon balm, either as dried plant or decoction, in "Serra da Estrela" cheese were analysed parameter by parameter, considering each species individually. Despite different significant variations, the identification of explicit tendencies is easier when all the parameters are evaluated simultaneously, including data for both species or for all natural additive type. Accordingly, two distinct linear discriminant analysis (LDA) were applied, where "plant species" and "natural additive type" were sequentially used as grouping factors. The significant independent variables (evaluated parameters) were selected using the stepwise procedure of the LDA, according to the Wilks' λ test. Only those with a statistical significant classification performance ($p < 0.050$) were kept in the analysis.

In the discriminant model designed to verify if the plant species (none, chestnut and lemon balm) had an overall influence in the evaluated parameters, the defined functions (plotted in **Figure 2**) integrated 100% of the observed variance (first: 57.3%; second: 42.7%). Among the tested variables, those selected as having discriminant ability were moisture, fat, protein, ash, Fe, Mg, K, Cu, Zn, C18:0, C20:3, C22:0, C22:6, C23:0 and C24:0, which indicates them as the most affected variables in function of the used plant species. Function 1 (more correlated with C22:0, Cu, Zn and ash) separated mainly the group corresponding to cheese samples with lemon balm; function 2 (more correlated with Fe, C20:3, C22:6, Mg and C24:0), on the other hand, separated mainly samples with chestnut flowers and control samples.

Regarding the effects of functionalizing type (independently of the plans species), the defined functions also included 100% of the observed variance (first: 74.6%; second: 25.4%), selecting moisture, fat, energy, Fe, Mg, Zn, Ca, K, C11:0, C13:0, C18:1, C18:2, C22:6, C24:0 and SFA as the significant discriminant variables. The markers

corresponding to each functionalizing type were completely separated (**Figure 3**). Regarding the variables more correlated to function 1 (Fe, C22:6, Ca and Mg), samples corresponding to the decoctions showed higher similarity to the control than the dried samples; on the other hand, dried samples were closer to the control samples for the variables more correlated to Function 2 (C24:0, C11:0 and C13:0).

4. Conclusion

When analyzed individually, the parameters reported herein showed some significant changes among control cheese and that added with dried plants or their decoctions (natural additives). Furthermore, there were evident changes among the initial stage and the end of maturation. The moisture loss was higher overtime in the samples incorporated with the plants, with a higher prevalence in the dried flowers. This increased the amount of the other parameters after the 6 months. The loss of water was desired, in order to decrease contamination probability and the proliferation of proteolytic bacteria, helping maintain the protein fraction of the cheese. In this particular type of cheese, maturated cheese is also very appreciated and bought at a higher price. By losing a higher quantity of moisture, the maturing could be significantly reduced. Furthermore, due to the loss of moisture, all other analyzed parameters seemed to have increased overtime, which was expected. Overall, the direct incorporation of dried plants (lemon balm or chestnut flowers) seemed to be a better alternative to the decoctions. The dried plants ensured a higher loss of moisture, lower calcium and sodium, higher preservation of the most abundant MUFA (C18:1) and higher quantity of PUFA (after 1 month). The decoctions, seemed to preserve the proteins in both of the analysed times. Although the preservation of proteins is very important for the organoleptic interest, the decoctions fall close behind of the preservations aspects of the dried plants, although quite better than the control samples. In certain cases, the color and

taste of the cheeses could be changed by incorporation of the dried plants, and therefore become less appealing to certain customers, while the incorporation of the decoctions went undetected. These conclusions are quite interesting, for one, the dried plants could be preserving agents while also lending taste and a different appearance to the cheese, which could interest some consumers but discourage others. On the other hand, the decoctions, although not so strong in the preserving area, could still carry this out while not changing the overall taste and appearance of the cheese. Some general conclusions were achieved through the performed LDA, which show the plant species (independently of natural additive type) and the natural additive type (independently of plant species) that better maintained the measured parameters in the control samples. By evaluating the produced outcomes, especially considering the correlations among selected variables and discriminant functions, it is also possible to suggest the best conditions to be applied in order to obtain a determined profile in the maturated "Serra da Estrela" cheese. Overall, the dried plant incorporations had higher preserving capacity when compared to the decoctions, but they also alter the appearance of the cheese. Thus, the decoctions, although slightly inferior in terms of preservations (only better for protein preservation) could also be good candidates for this functions due to not altering the appearance of cheese. Still, in terms of promoting differentiated foods (different colors and taste), the dried plants proved to be better.

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Conflict of Interest

The authors state that there is no conflict of interest regarding this manuscript.

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Figure 1. Estimated marginal mean plots representing the effects of NA and ST on moisture (A), fat (B), protein (C), Ca (D), Na (E), C16:0 (F), C18:1 (G), SFA (H), MUFA (I) and PUFA (J).

Figure 2. Mean scores of cheese samples with plant species projected for the two discriminant functions defined from all evaluated parameters.

Figure 3. Mean scores of cheese samples with natural additive type projected for the two discriminant functions defined from all evaluated parameters.

Table 1. Proximate composition and energy value of cheese samples added with chestnut flowers or lemon balm (dry material or lyophilized decoction as natural additives- NA) further submitted to storage (ST). The results are presented as mean±SD[1].

		Moisture (g/100 g fw)	Fat (g/100 g fw)	Proteins (g/100 g fw)	Ash (g/100 g fw)	Energy (kcal/100 g fw)
Chestnut flowers						
NA	None	43±7	26±2	33±4	4±1	367±31
	Dried plant	39±11	27±5	35±5	4±1	379±63
	Decoction	40±10	26±3	36±6	5±1	380±47
	p-value (n=18)	0.355	0.924	0.171	<0.001	0.684
ST	1month	50±1	23±1	30±1	4±1	330±13
	6 months	31±4	29±1	39±2	5±1	420±19
	p-value (n=36)	<0.001	<0.001	<0.001	<0.001	<0.001
NA×ST	*p*-value (n=72)	<0.001	<0.001	<0.001	<0.001	<0.001
Lemon balm						
NA	None	43±7	26±2	33±4	4±1	367±31
	Dried plant	38±10	26±3	35±4	5±1	375±49
	Decoction	43±6	25±2	33±3	5±1	356±33
	p-value (n=18)	0.105	0.206	0.296	<0.001	0.338
ST	1 month	49±1	23±1	30±1	4±1	330±7
	6 months	34±4	28±1	37±1	5±1	402±16
	p-value (n=36)	<0.001	<0.001	<0.001	<0.001	<0.001
NA×ST	*p*-value (n=72)	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2. Minerals profile (mg/100 g fw) of cheese samples added with chestnut flowers or lemon balm (dry material or lyophilized decoction as natural additives- NA) further submitted to storage (ST). The results are presented as mean±SD[1].

		Ca	Mg	Na	K	Fe	Zn	Cu
				Chestnut flowers				
NA	None	987±158	62±13	1193±384	177±59	0.6±0.3	6±1	0.4±0.1
	Dried plant	1289±149	81±13	1225±371	209±79	1.3±0.4	7±1	0.4±0.1
	Decoction	1150±294	70±18	1249±480	174±80	1.1±0.1	7±1	0.3±0.1
	p-value (n=18)	<0.001	0.001	0.921	0.298	<0.001	<0.001	0.148
ST	1 month	948±143	57±9	823±36	116±15	0.9±0.1	6±1	0.5±0.1
	6 months	1335±142	85±9	1621±82	257±22	1.0±0.5	7±1	0.3±0.1
	p-value (n=36)	<0.001	<0.001	<0.001	<0.001	0.385	<0.001	<0.001
NA×ST	*p*-value (n=72)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
				Lemon balm				
NA	None	987±158	62±13	1193±384	177±59	0.6±0.3	6±1	0.4±0.1
	Dried plant	1189±140	70±9	1691±656	205±71	1.5±0.5	6±1	0.2±0.1
	Decoction	958±117	65±9	1212±299	177±52	1.2±0.2	5±1	0.3±0.1
	p-value (n=18)	<0.001	0.061	0.003	0.293	<0.001	0.028	<0.001
ST	1 months	921±97	56±5	932±98	127±7	1.0±0.1	4±1	0.4±0.1
	6 months	1167±138	76±3	1799±384	245±21	1.2±0.5	6±1	0.2±0.1
	p-value (n=36)	<0.001	<0.001	<0.001	<0.001	0.115	<0.001	<0.001
NA×ST	*p*-value (n=72)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 3. Fatty acids profile (relative percentage) of cheese samples added with chestnut flowers or lemon balm (dry material or lyophilized decoction as natural additives- NA) further submitted to storage (ST). The results are presented as mean±SD[1].

		C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C15:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	SFA	MUFA	PUFA
Chestnut flowers																		
NA	None	2.3±0.1	2.6±0.2	2.6±0.3	8±1 ab	5±1	11±1	1.4±0.2	25±2	1.0±0.1	1.0±0.1	11±1 a	23±4	2.1±0.4	1.8±0.2	71±4	24±3	4.8±0.5
	Dried plant	2.0±0.1	2.3±0.2	2.3±0.2	7±1 b	5±1	11±1	1.5±0.2	26±2	0.9±0.1	1.1±0.1	11±1 a	24±4	2.2±0.4	1.8±0.1	70±4	25±4	4.9±0.5
	Decoction	3.0±0.1	2.9±0.2	2.9±0.4	8±1 a	5±1	11±1	1.4±0.2	25±1	0.9±0.1	1.1±0.1	10±1 b	22±4	2.2±0.2	1.7±0.2	72±4	23±3	4.6±0.5
	p-value (n=18)	<0.001	<0.001	<0.001	0.002	0.266	0.912	0.901	0.178	0.098	0.407	<0.001	0.612	0.825	0.088	0.522	0.576	0.264
ST	1 month	2.3±0.2	2.5±0.2	2.4±0.2	7±1	4.3±0.3	10.6±0.4	1.3±0.1	24±1	0.9±0.1	1.0±0.1	11±1	26±1	2.5±0.2	1.9±0.1	67±1	28±1	5.3±0.3
	6 months	2.4±0.4	2.6±0.4	2.9±0.4	9±1	5.6±0.3	12.5±0.4	1.6±0.1	27±1	1.0±0.1	1.1±0.1	10±1	19±1	1.9±0.2	1.7±0.2	75±1	21±1	4.3±0.2
	p-value (n=36)	0.086	0.249	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
NA×ST	*p*-value (n=72)	<0.001	0.001	0.001	0.244	0.553	0.009	0.199	0.003	0.040	<0.001	0.215	0.785	<0.001	0.611	0.835	0.999	0.170
Lemon balm																		
NA	None	2.3±0.1	2.6±0.2	2.6±0.3	8±1	5±1	11±1	1.4±0.2	25±2	1.0±0.1 a	1.0±0.1	11±1	23±4	2.1±0.4	1.8±0.2	71±4	24±3	4.8±0.5
	Dried plant	2.0±0.2	2.3±0.3	2.3±0.2	7±1	5±1	11±1	1.5±0.2	26±2	1.0±0.1 a	1.1±0.2	11±1	24±4	2.1±0.4	1.9±0.2	70±5	25±4	4.8±0.5
	Decoction	2.6±0.5	2.7±0.5	2.8±0.5	8±1	5±1	11±1	1.4±0.2	25±1	0.9±0.1 b	1.0±0.1	11±1	23±5	2.3±0.3	1.8±0.2	71±5	24±5	4.8±0.5
	p-value (n=18)	<0.001	0.003	0.006	0.133	0.974	0.783	0.426	0.249	0.001	0.078	0.250	0.735	0.584	0.334	0.771	0.716	0.991
ST	1 month	2.1±0.2	2.4±0.2	2.3±0.2	6±1	4.2±0.3	10.5±0.4	1.3±0.1	24±1	0.9±0.1	1.0±0.1	11±1	27±1	2.6±0.2	2.0±0.2	66±1	28±1	5.4±0.3
	6 months	2.5±0.5	2.6±0.4	2.9±0.4	8±1	5.6±0.3	12.5±0.4	1.6±0.1	27±1	1.0±0.1	1.1±0.1	10±1	19±1	1.8±0.2	1.7±0.2	75±1	21±1	4.2±0.2
	p-value (n=36)	0.001	0.053	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
NA×ST	*p*-value (n=72)	<0.001	<0.001	<0.001	<0.001	0.010	0.044	0.439	<0.001	0.191	<0.001	<0.001	<0.001	0.093	0.433	<0.001	<0.001	0.009

[1]Means within a column with different letters differ significantly ($p < 0.05$).